Redox Responsive Pluronic Micelle Mediated Delivery of Functional siRNA: A Modular Nano-Assembly for Targeted Delivery

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Experimental section

General

All chemicals, reagents and dry solvents for Pluronic and RNA synthesis were purchased from Sigma–Aldrich (Sweden) and used without any further purification. Unmodified phosphoramidites (N^6-benzoyl-rA, N^2-isobutyl-rG, N^4-acetyl-rC, rU and dT) and solid supports were purchased from ChemGene Corporation (USA). ^{1}H NMR was recorded on 400 MHz instruments (Jeol JNM-ECP Series FT NMR). The chemical shifts in parts per million (δ) are reported downfield from TMS (0 ppm).

Preparation of pyridyl disulfide ligand (2)

β-Mercaptoethylamine (cysteamine) hydrochloride (2.288 g, 20.14 mmol) **1** was dissolved in methanol (MeOH, 17.5 mL) followed by addition of glacial acetic acid (1.6 mL). This solution was then added dropwise to a stirred solution of 2,2-dithiopyridine (8.815 g, 40.28 mmol) in methanol (41.6 mL). The reaction was stirred for 48 h at room temperature, and the product was precipitated from stirred diethyl ether (200 mL). The precipitated product was re-dissolved in a small volume of methanol and was precipitated again by diethyl ether. The final product was purified with silica gel column chromatography (10% MeOH/ ethyl acetate) to afford white solid compound **2** (3.3 g, 73 %). ^{1}H NMR (400 MHz, D_2O) δ ppm 8.31 - 8.50 (m, 1 H) 7.78 - 7.84 (m, 1 H) 7.70 - 7.75 (m, 1 H) 7.28 - 7.33 (m, 1 H) 3.30 - 3.35 (m, 3 H) 3.06 - 3.11 (m, 2 H).

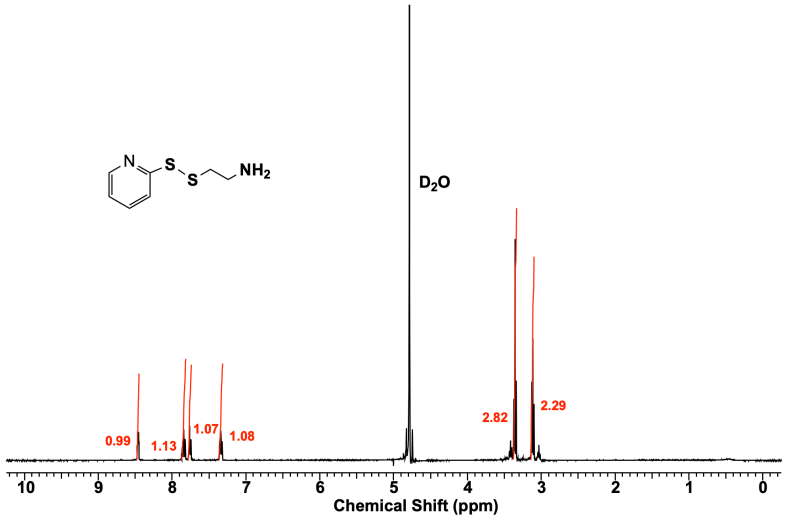


Figure S1. ^{1}H NMR spectra for ligand **2**

Preparation of activated pluronic with 4-nitrophenyl chloroformate (4)

Pluronic F108 (2 g, 0.14 mmol) was dissolved in 10 mL of dichloromethane (DCM), and to this solution, 4-nitrophenyl chloroformate (170 mg, 0.84 mmol) was added slowly. The reaction mixture was stirred overnight at room temperature, and the product was precipitated with diethyl ether. Precipitation was repeated for three times to afford **4** with 99% yield (2.025 g). The degree of modification was determined using ultra-violet (UV) spectroscopy at 402 nm and employing a molar

extinction coefficient of 18400 cm^{-1} M^{-1}. For this purpose, activated pluronic was hydrolyzed by dissolving in 0.1 M NaOH at a concentration of 1 mg/mL. After 1h, the absorbance was measured, which indicated a near quantitative activation of hydroxyl groups (~100%).

Synthesis of F108 pyridyl disulfide pluronic (5)

The pluronic derivative **4** (1 g, 0.07 mmol) was dissolved in 10 mL of DCM. Before adding ligand **2** to the reaction mixture, the hydrochloride salt was neutralized, and for this purpose, **2** (154 mg, 0.69 mmol) was extracted with DCM and saturated $NaHCO_3$ and then dried over Na_2SO_4 followed by vacuum concentration to get the yellowish oily compound. Subsequently, the extracted ligand was dissolved in 1 mL of DCM and added to the above reaction mixture. The reaction mixture was refluxed for overnight. The reaction mixture was concentrated and then diluted with methanol:water (1:1, v/v, 10 mL) and dialyzed (1000 or 3500 Da cutoff) against 2 L of deionized water for two days. Product **5** was finally recovered by lyophilization to get white solid (0.958 g, 92%). The degree of modification was determined using UV employing 343 nm, and molar extinction coefficient of 8060 cm^{-1} M^{-1}. The UV absorbance of the product (dissolved as 1 mg/ mL in PBS buffer, pH 9) was measured before as well as 10 min after the addition of 0.1 mL of DTT (dissolved as 15 mg/mL in PBS, pH 9) demonstrating nearly quantitative conversion (~100%).

Synthesis of pluronic-siRNA conjugates with disulfide linkage (6)

Disulfide functionalized siRNA was synthesized on automated solid-phase synthesizer employing standard synthesis cycle for RNA. In the case of the sense strand, thio-modified solid support was utilized for synthesis. Further, both the strands were deprotected and purified by denaturing PAGE. Thereafter RNA samples were recovered with TEN buffer, and pure RNA pellets were dissolved in water. Equimolar amounts of both the strands were mixed and heated at 95 °C for 2 min, then gradually cooled to room temperature over a period of 3 h, and stored at 4 °C to get the siRNA duplexes. To the solution of duplex RNA (50 µL, 2.5 nmol, 25 µM), DTT (10 µL, 50 mM) and H_2O

(40 µL) were added. The reaction mixture was incubated at 37 °C for 2 h. Further 3 M NaCl (150 µL) was added, followed by H_2O (150 µL) and the mixture was vortexed and spun down. EtOH (100%, 1000 µL) was added, vortexed and kept in –20 °C for overnight. The RNA was micro-centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was removed. The pellet was washed with absolute EtOH (100 µL), micro-centrifuged at 13,000 rpm at 4 °C for 10 min and the supernatant was removed. The pellet was directly dissolved in a solution of **5** (368 µL, PBS, pH 8, 1250 pmol). The reaction mixture was incubated overnight at room temperature. Then the reaction mixture was directly used for conjugation analysis and gene knockdown experiments. The yield of RNA conjugate **6** was ~95% as determined by gel electrophoresis.

Synthesis of pluronic- peptide-siRNA (P-SS-STAT3-COOP) conjugates with disulfide linkage

The COOP peptide having N-terminal cysteine (CACGLSGLGVA) was conjugated to the Pluronic surface by thiol-exchange reaction utilizing pyridyl disulfide group in Pluronic F108. Briefly, 0.5 µL of peptide (6 mM) was added to the 135 µL of functionalized Pluronic solution (2 mg/100 µL) in PB (100 mM, 150 mM NaCl, pH 8) and incubated for 30 minutes at room temperature. This corresponds to 0.8% pyridyl substitution of the modified Pluronic F108, assuming quantitative coupling. Thereafter, 20 µL of thiol-modified (DTT treated) STAT3 siRNA (100 µM) was added to 12.5 µl of above-mentioned peptide-Pluronic conjugate, incubated overnight at room temperature and directly used for the knockdown experiments.

Polyacrylamide gel electrophoresis assay

The conjugated siRNA was mixed with 5 µL of 2X stop dye (10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60 % glycerol and 60 mM EDTA) and loaded on the gel. The samples were analyzed by 15% native PAGE (0.7 mm thickness, 15-20 W) with 1X TBE (89

mM of each tris and boric acid, 2 mM of EDTA, pH 8.3) as running buffer. Gels were stained with SYBR® Green (Thermo Fischer scientific, Sweden) stain for 15 minutes and visualized by UV lamp.

Particle size distribution by dynamic light scattering

The hydrodynamic size of the micelles was estimated using Malvern laser granulometer (Zetasizer Nano ZS, Malvern, United Kingdom). Briefly, 100 nM equivalents of siRNA in P-SS-STAT3 was added to 50 µL of 100 mM $CaCl_2$. This mixture was then vortexed and incubated at room temperature for 10 min. 700 µL of deionized water was added to the solution. For the group without the calcium complexation, 100 nM equivalents of siRNA in P-SS-STAT3 was taken and added directly to 750 µL of deionized water. Both the formulations were then filtered using 0.45 µm filter before performing the DLS experiment. Thereafter, the solutions were placed in a disposable PMMA cuvette and the DLS measurements were recorded at 25 °C. The same concentrations of siRNA were also used for P-SS-STAT3-COOP and P-SS-STAT3-COOP/Ca.

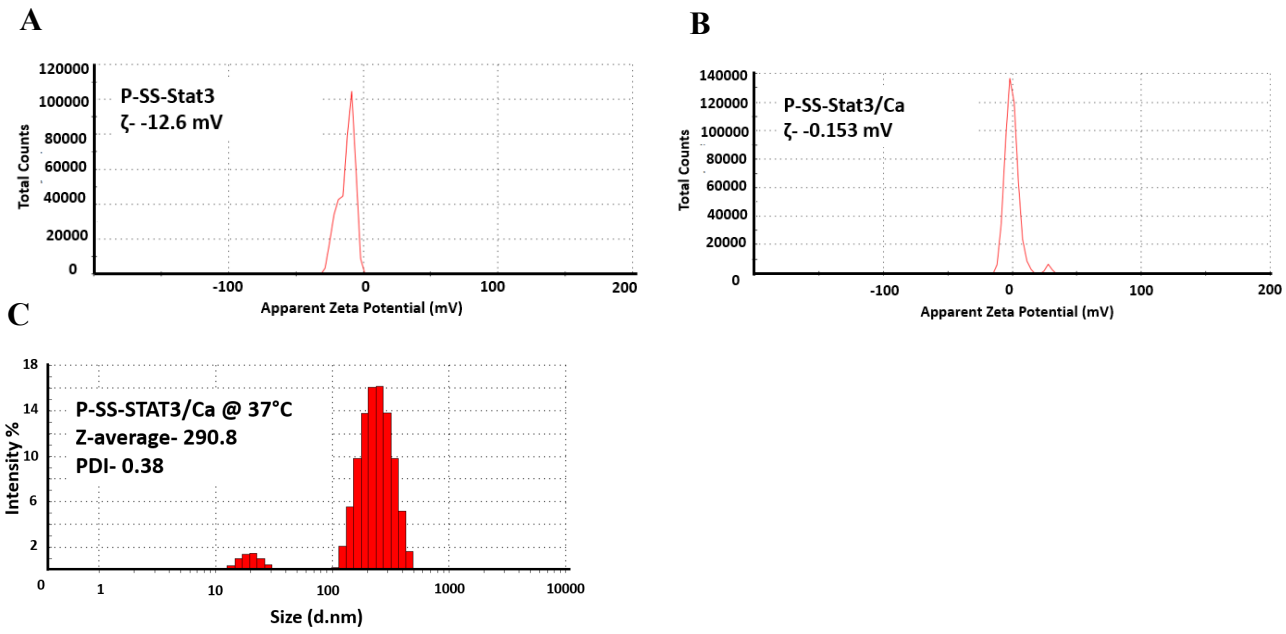


Figure S2. Zeta potentials of (A) P-SS-STAT3 and (B) P-SS-STAT3/Ca. (C) Hydrodynamic size of the Plu-SS-STAT3/Ca after incubation at 37°C.

Scanning Electron microscopy

The morphology of the Plu-SS-TF/Ca nanoparticles was determined using an SEM (scanning electron microscope) (Zeiss LEO 1550) operating at 5 kV using the Inlens detector. Prior to the analysis, the samples were diluted in deionised water and drop casted on to the carbon tabs (Agar Scientific, Stanstead, UK). The samples were then allowed to dry at room temperature in a desiccator overnight. As the samples were non-conductive, a thin layer of gold was evaporated on to the samples to make them suitable for SEM analysis.

Cell culture conditions

The human cell lines, namely, HCT116, U87 and HDF were obtained from ATCC and were cultured in DMEM high glucose (Thermo Fischer Scientific, Sweden) with 10% FBS and 1% Pen-Strep. For qPCR experiments, 50000 cells per well were seeded in a 24 well plate, and for western blot analysis, 200000 cells were plated in a 6-well plate for 24 hours in 37 °C incubator before transfection.

Patient-derived BT-13 gliospheres were cultured in Corning Ultra-low cell culture dishes (Sigma) with DMEM/F12, Glutamax (Thermo Fischer Scientific), 1.5% 1 M Hepes (Gibco) and 2% B27 supplement 50X (Gibco). These cells were also cultured under serum conditions where 10% FBS was added in addition. The hanging drop method was used to prepare spheroids with 40,000 cells and were cultured in suspension and left overnight in an incubator at 37 °C with 5% CO_2.

For experiments involving embryonic stem cells, feeder-independent E14 mouse embryonic stem cells from (mES) cells were cultured in 2i medium in a serum-free N2B27 medium[27] supplemented with MEK inhibitor PD0325901 (1 μM) and GSK3β inhibitor CHIR99021 (3 μM) (Selleckchem), and 1000 U/ml LIF (Millipore). TrypLE™ Express (Life Technologies) was used to passage the cells.

The 2i media was supplemented with 2% ES-qualified FBS (Thermo Fischer Scientific) when seeding the cells.

MDGI expression levels

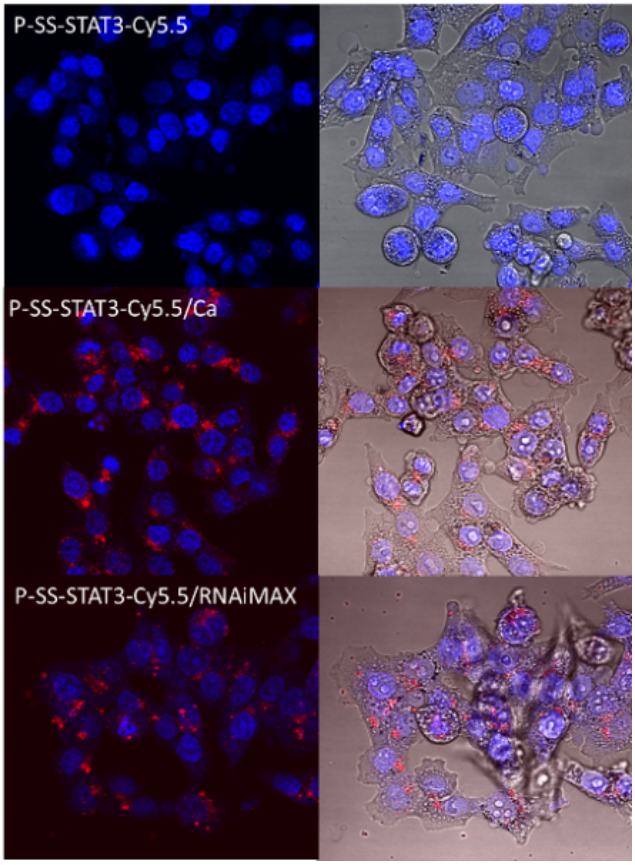
The BT-13 cells were cultured 40,000 in number on a 24 well plates in presence of serum in conditions mentioned above. The cells in serum free conditions (40,000) in number were grown as spheroids as mentioned above. These cells were incubated for a period of three days after which RNA was isolated using the RNeasy Plus Mini kit from Qiagen. The cDNA was prepared using High capacity RNA to cDNA kit (Applied Biosystems) according to manufacturers protocol. qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix from BioRad. The PCR reactions were carried out with 10 µL of SsoAdvanced Universal SYBR Green Supermix, 2µL cDNA, and 0.4 µL of forward primer and 0.4 µL of reverse primer (STAT3 and β-actin (*ACTB*)) in a 20 µL final reaction volume. Reference gene, β-actin was selected as a control for normalization of real-time PCR data. The amplification was carried out using the Biorad CFX connect system (Bio-rad, Sweden) using a 40-cycle program. The CFX manager software automatically calculates the raw Ct (cycle threshold) values. Samples were normalized relative to endogenous control and differences in cycle number thresholds were calculated using comparative quantitation $2^{-\Delta\Delta CT}$ method (also called the ΔΔCT method).

Transfection experimental condition

Cells were transfected with pluronic F108 conjugated siRNA (compound **6)** that targets STAT3, OCT4 and cy5.5-labelled STAT3, with calcium chloride ($CaCl_2$) and RNAiMAX (Thermo Fischer Scientific). Briefly, P-SS-STAT3/Ca or P-SS-OCT4/Ca were prepared by adding 50 nM of P-SS-STAT3 or P-SS-OCT4 to 25 µL of 100 mM $CaCl_2$. They were mixed by vortexing followed by incubation at room temperature for 10 minutes. After incubation, the P-SS-STAT3/Ca or P-SS-OCT4/Ca were added to cells in a 24 well plate. Cells were also transfected with P-SS-STAT3 or P-

SS-OCT4 using RNAiMAX as a positive control. Unconjugated STAT3 siRNA were transfected with both RNAiMAX and $CaCl_2$ using identical amounts of siRNA and $CaCl_2$ as in the experiment mentioned above. The cells were incubated for 24 hours after transfection at 37 °C incubator having 5% CO_2.

A



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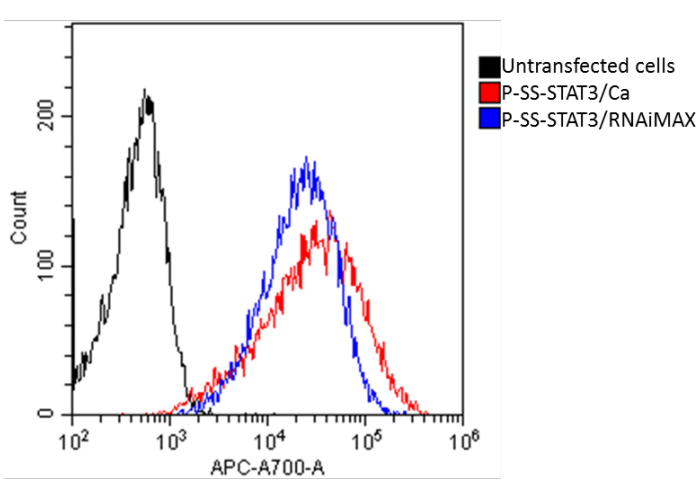

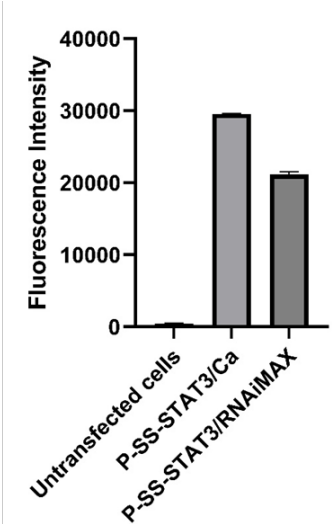

Figure S3. (A) Images of HCT116 cells were treated with a) P-SS-STAT3-Cy5.5, b) P-SS-STAT3-Cy5.5/Ca and c) P-SS-STAT3-Cy5.5/RNAiMAX showing perinuclear localization of siRNA.(B) Flow cytometry of cells treated with P-SS-STAT3-Cy5.5/Ca and P-SS-STAT3-Cy5.5/RNAiMAX.

Quantitative real-time-PCR

After 24 hours post-transfection, the cells were lysed, and RNA was extracted. In each experiment, 1 µg of the total RNA was used to make the cDNA. The cDNA was prepared using High Capacity RNA to cDNA kit according to manufacturer's protocol (Applied Biosystems, USA) and qRT-PCR was performed with cDNA and TaqMan® Fast Universal PCR Master Mix (2X) (Applied Biosystems, USA). The real-time PCR reactions were carried out with 10 µL of 2x TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Applied Biosystems, USA), 5 µL of 1:5 diluted cDNA, and 2 µL of TaqMan gene-specific assay mix (STAT3 and β-actin (*ACTB*))(Applied Biosystems, USA) in a 20 µL final reaction volume. Reference gene, β-actin (Taqman primers, Thermo Fischer Scientific, Sweden) were selected as a control for normalization of real-time PCR data. The amplification was carried out using the Biorad CFX connect system (Bio-rad, Sweden) using a 40-cycle program. The CFX manager software automatically calculates the raw Ct (cycle threshold) values. Data from samples with a Ct value equal to or below 30 were further analyzed. Samples were normalized relative to endogenous control and differences in cycle number thresholds were calculated using comparative quantitation $2^{-\Delta\Delta CT}$ method (also called the ΔΔCT method), which is commonly used for analyzing siRNA induced gene knockdown efficiency. The formulas used to calculate gene knockdown were as follows:

First, the ΔCT was calculated as the mean cycle threshold for the target gene minus the mean cycle thresholds for the endogenous controls *ACTB*, each performed in triplicates:

ΔCT = CT (target gene) – CT (endogenous control). Secondly, the ΔΔCT was calculated as the ΔCT of the target minus the ΔCT of a negative control (NC): ΔΔCT = ΔCT (target) – ΔCT (NC). Thereafter, the percentage of knockdown of the target gene was calculated as:

Fold change = 2−ΔΔCT, then percentage of Knockdown: = 100 * (1-fold change)

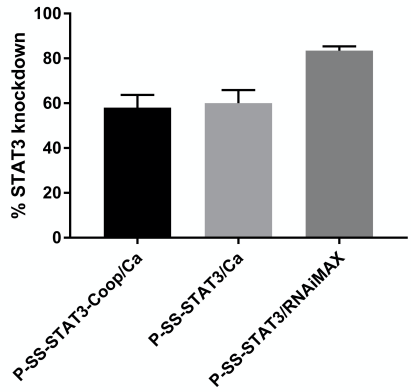

Figure S4. Transfection efficiency of the P-SS-STAT3-COOP/Ca and P-SS-STAT3/Ca nanoparticles in patient-derived BT-13 cells in presence of serum (2D culture).

Western blotting

After 48 h of transfection, cells were washed twice with ice-cold PBS and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, and 1% protease inhibitor cocktail from SIGMA-ALDRICH®). Lysates were incubated on ice for 30 minutes and centrifuged at 10,000 rpm for 20 minutes to collect the supernatant. Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fischer Scientific, Sweden) was used to measure protein concentrations. Thereafter, 20 µg of soluble protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Primary antibodies against STAT3 (1:1000 dilution Cell Signaling Technology®), β-actin (1:1000; Cell Signaling Technology®) were used to probe the protein bands. Anti-Rabbit-HRP conjugated secondary antibodies (1:3000 dilution; Biorad®) were used to detect the primary antibodies, followed by the target protein visualization with Clarity Chemiluminescent Substrate (ECL). Images were acquired using ChemiDoc XRS.

Cell viability assay

After 48 h post-transfection, the medium in the cells were replaced with 10% alamarBlue (Thermo Fischer Scientific, Sweden) in cell culture medium for measuring the viability of transfected cells.

The cells were then kept in 37 °C incubator for 2 hours. The medium was then transferred to a black plate for fluorescence readout using a Tecan microplate reader. The values were normalized to untreated cells.

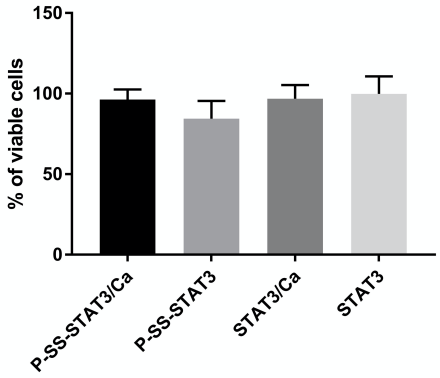

Figure S5. AlamarBlue assay for toxicity in HCT116 cells.

Confocal imaging

24 Hours post-transfection, HCT116 cells were fixed with 4% formaldehyde and washed with PBS thrice and stained with Hoechst 33342 (Thermo Fischer Scientific, Sweden) for 10 mins and washed with PBS thrice and imaged by ZEISS LSM 710 confocal microscope at 20X.

Flow Cytometry

24 Hours post-transfection, HCT116 cells were trypsinised and resuspended in PBS. Cytoflex S was used to analyze the cells. Cells were gated using FSC-A and SSC-A. The gated cells were further gated for single cells using FSC-H and FSC-A. APC-A700 channel was used to detect Cy5.5. Median fluorescent intensity was calculated for all the cells.